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Selective Induction of DNA Synthesis in Mouse Preneoplastic and Neoplastic Hepatic Lesions after Exposure to Phenobarbital

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Recent evidence has suggested that the induction of DNA synthesis by nongenotoxic chemical carcinogens plays an important role in the formation of cancer. The present study examined the effect of a nongenotoxic carcinogen, phenobarbital, (PB) on the induction of DNA synthesis in preneoplastic foci and adenomas in B6C3F$_1$ mice. Male mice were treated with diethylnitrosamine at 30 days of age. After 6 months, mice had both hepatic foci and adenomas. Mice were divided into three groups at random and treated with PB in the drinking water and examined for DNA labeling by autoradiography. Before sacrifice, each mouse received an osmotic minipump containing [^{3}H] thymidine. Results showed a PB dose-dependent increase in DNA synthesis in hepatic foci. Adenomas were unresponsive to the DNA synthesis-enhancing effect of PB, maintaining a level of 20-25%. The normal surrounding liver showed an increase in DNA synthesis (10–15% labeling index) at the 7-day sampling, which returned to normal control levels by 28 and 45 days. The foci showed a heterogeneity in response, with some foci showing an increase (20–30% labeling index), and others maintaining control DNA synthesis levels (4–6% labeling index). These results show that preneoplastic foci in the mouse respond preferentially to the induction of DNA synthesis by PB, that this response is dose dependent, and that it is maintained as long as the treatment continues.

Introduction

The development of liver cancer in rodents after exposure to chemical carcinogens is a multistep process (*1–3*). In the liver, at least three stages, initiation, promotion, and progression, have been identified using biochemical and morphological markers. DNA synthesis and cell proliferation are important in each of these stages. During the initiation stage, a single liver cell is genotypically modified. Cell proliferation is necessary in this stage to lock in this mutational change. The stage of tumor promotion involves the selective clonal expansion of the initiated cell to a focal group of cells. This change appears to be reversible and is histologically represented in the liver by the hepatic focal areas of cellular alteration [focus (*4*)]. The progression stage also involves a genotoxic, mutational event. In the liver, the progression stage is represented histologically by the appearance of neoplastic lesions (adenomas and carcinomas). Cell proliferation is necessary for the expansion and growth of the neoplastic lesions (*1–5*).

A number of chemical carcinogens appear to induce hepatic tumors in rodents through nongenotoxic mechanisms. The mechanism(s) by which these compounds induce cancer in the liver of rodents remains unresolved. Nongenotoxic carcinogens are generally negative in short-term genotoxicity, mutation, and cell transformation assays. What is apparent is that the nongenotoxic hepatic carcinogens exhibit a number of similar cellular changes in the rodent liver after exposure. These include induction of mixed-function oxidase enzymes including P450, inhibition of gap junctional intercellular communication, induction of hepatocyte DNA synthesis, and induction of hepatocyte cell proliferation. These latter two parameters have recently received extensive attention. Two possible mechanisms by which the induction of DNA synthesis and cell proliferation by these nongenotoxic carcinogens may induce hepatic cancer have been put forth (*6*). In one scenario, the induction of hepatic DNA synthesis has been proposed to produce genetic mutation through the upsetting of normal DNA synthesis and replication. One of three fates can beset these

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mutated cells: the DNA damage is repaired, the mutation is incompatible with life and cell death occurs, or the mutation is not repaired, producing an initiated cell. In the latter outcome, the initiated cell then has the potential to progress through the stages of hepatic tumorigenesis, eventually resulting in a neoplasm.

In a second scenario, the liver already contains a number of initiated cells (either spontaneously induced or from exposure to an initiating agent) that require the additional events to allow these cells to progress to the neoplastic stage. In this case, exposure to a nongenotoxic carcinogen may result in the selective proliferation and clonal expansion of the initiated cells. This would result in the formation of hepatic foci with the possibility of some of the foci progressing to hepatic adenomas and carcinomas. This selective induction of DNA synthesis by the nongenotoxic carcinogen may be the result of differences in the preneoplastic cell that allow for the increased proliferative response, an inhibition of DNA synthesis in the normal surrounding liver, or a combination of both effects. In the current study, the role that cell proliferation may play in this second scenario was investigated in the mouse liver after exposure to the nongenotoxic carcinogen phenobarbital.

Methods

Male B6C3F$_1$ mice were used in this study. Mice were purchased from Charles River Breeding Laboratories at 21 days of age. After 7 days of acclimation, mice were treated with a single IP injection of diethylnitrosamine (DEN) at 30 days of age (90 mg/kg BW (*7*). Mice were then maintained on Purina Lab Blox and deionized water *ad libitum* for 36 weeks. At 36 weeks, mice were randomly divided into four groups of 12 mice each. Group 1 received 500 mg/L of phenobarbital in their drinking water; group 2 received 100 mg/L phenobarbital in their drinking water; group 3 received 20 mg/L of phenobarbital in their drinking water; and group 4 received 0 mg/L of phenobarbital in their drinking water. Mice were treated with phenobarbital for 7, 14, 28, and 45 days. Three mice from each group were sacrificed at each time period. Seven days before sacrifice, each mouse received an osmotic minipump containing [^{3}H] thymidine (*8*). At sacrifice, livers were weighed and processed for histology. Paraffin-embedded liver from each mouse was sectioned, processed for autoradiography, and stained with hematoxylin and eosin. The labeling index for hepatic foci, adenomas, and normal surrounding liver was determined by counting the number of labeled hepatocytes (*7,8*). In foci and adenomas, all of the hepatocytes within the lesion on the histological section were scored for DNA synthesis. In the normal surrounding liver, randomly selected areas of 50 cells were scored. Hepatic foci were histologically grouped as a class, and unique tinctorial characteristics (i.e., basophilic, eosinophilic, clear cell) were not used to differentiate the foci because previous studies have shown that mouse liver foci treated with phenobarbital tend to express the eosinophilic phenotype during the phenobarbital treatment. A total of 50 foci, 25 adenomas, and 1500 normal, surrounding hepatocytes were quantitated for labeling index from the three mice for each sampling group and sampling time. Labeling index values were statistically compared by Fisher's Exact test (*7,8*).

Results

The normal, surrounding liver showed a dose-dependent increase in DNA synthesis at the 7-day and 14-day sampling times, which returned to levels of untreated controls by the 28-day sampling time. (Fig. 1). Hepatocytes from untreated control mice showed an average labeling index of 4–5% at each of the four sampling times. The induction of DNA synthesis by phenobarbital in the normal, surrounding hepatocytes was dose dependent. Treatment with 500 mg/L phenobarbital in the drinking water produced a labeling index of 19.4% after 7 days and 22.5% after 14 days, whereas treatment with 100 mg/L phenobarbital resulted in a labeling index of 14.4% after 7 days and 12.3% after 14 days. Treatment with 20 mg/L phenobarbital did not increase the labeling index over that of the untreated control.

A dose-dependent increase in DNA synthesis as measured by labeling index was also seen in hepatic foci from phenobarbital-treated mice (Fig. 2). Foci from untreated mice showed an average labeling index of 17.2% at the 7-, 14-, 28-, and 45-day sampling times. Treatment with 500 mg/L phenobarbital resulted in a significant increase in labeling index above the untreated control values. The labeling index in these

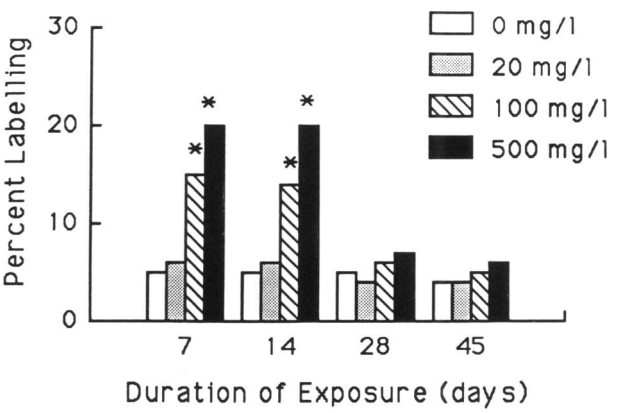

FIGURE 1. Labeling index in normal hepatocytes after treatment of B6C3F$_1$ male mice with phenobarbital at concentrations of 0, 20, 100 and 500 mg/L in the drinking water for 7, 14, 28, and 45 days. Three mice at each phenobarbital concentration were sampled at each time, and the labeling index was determined by autoradiography. A minimum of 500 cells were scored for each mouse. (*)Values significantly greater than the untreated control ($p < 0.05$).

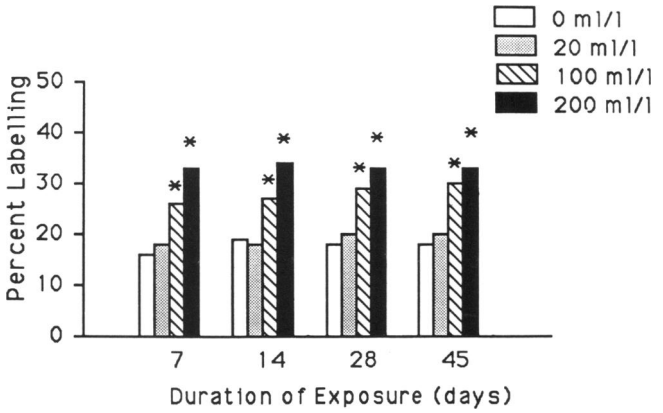

FIGURE 2. Labeling index in hepatocytes in foci after treatment of B6C3F$_1$ male mice with phenobarbital at concentrations of 0, 20, 100, and 500 mg/L in the drinking water for 7, 14, 28, and 45 days. Liver lesions were induced in the mice by treatment with a single dose of diethylnitrosamine at 30 days or age 36 weeks before treatment with the phenobarbital. Three mice at each phenobarbital concentration were sampled at each time, and the labeling index was determined by autoradiography. A minimum of 50 focal lesions were scored for the three mice. All cells in each focal lesion were scored. (*) Values significantly greater than the untreated control ($p < 0.05$).

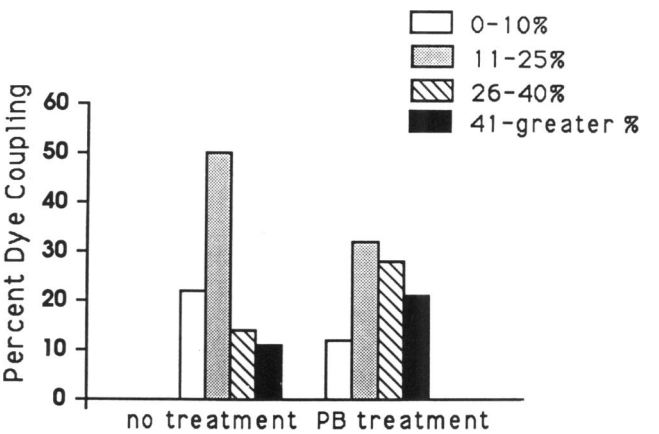

FIGURE 3. Labeling index in hepatic foci receiving either no phenobarbital or 500 mg/L phenobarbital for 7 days. Liver lesions were induced in the mice by treatment with a single dose of diethylnitrosamine at 30 days of age 36 weeks before treatment with the phenobarbital.

foci averaged 32.5% labeling for the 7-, 14-, 28-, and 45-day sampling times. Unlike the normal surrounding hepatocytes, the labeling index in the foci treated with 500 mg/L phenobarbital remained at a significantly increased level during the entire treatment period (Fig. 2). Treatment with 100 mg/L also produced a sustained increase in labeling index throughout the exposure period. The labeling index for the 100 mg/L treatment averaged 26.7% for the four sampling times (Fig. 2). This increase was significantly increased over control values and significantly lower than the labeling index values seen for the 500 mg/L treatment. Treatment of mice with 20 mg/L did not induce an increase in labeling index over the untreated control values at any of the times sampled (Fig. 2).

The DNA induction effect of phenobarbital in hepatic foci displayed a heterogeneous pattern of response (Fig. 3). When hepatic foci treated with 500 mg/L of phenobarbital were grouped based on their labeling index, it became apparent that not all of the hepatic foci responded to the phenobarbital effect on DNA synthesis in a similar manner. In untreated control mice (not receiving phenobarbital), the foci labeling index in more than 70% of the foci examined was 25% or less (Fig. 3). After treatment with phenobarbital for 7 days, a shift in the pattern of the labeled foci was seen, with over 50% of the foci exhibiting a labeling index of 26% or greater. These results suggest that there are several populations of hepatic foci with regard to DNA synthetic inductive response to phenobarbital.

Adenomas from mice treated with phenobarbital were unresponsive to the DNA synthetic-enhancing effects of this drug (Fig. 4). Adenomas showed a constant labeling index ranging from 21.4%–25.7% at all of the doses and sampling times of phenobarbital treatment.

Discussion

These results show that preneoplastic foci in the mouse respond preferentially when compared to normal hepatocytes to the induction of DNA synthesis by phenobarbital. This response is dose dependent, with the two highest doses of phenobarbital (500 mg/L and 100 mg/L) producing an increase in labeling index, and

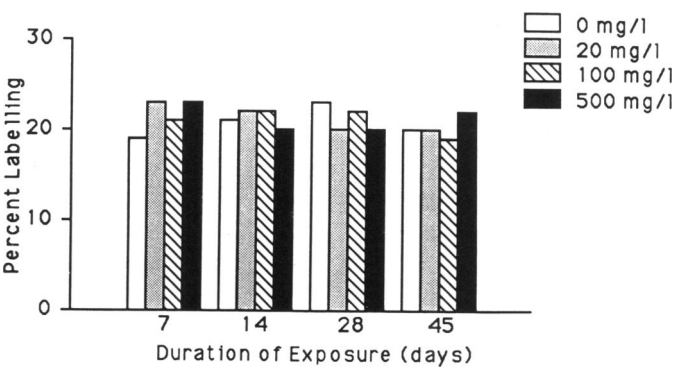

FIGURE 4. Labeling index in hepatocytes in adenomas after treatment of B6C3F$_1$ male mice with phenobarbital at concentrations of 0, 20, 100, and 500 mg/L in the drinking water for 7, 14, 28, and 45 days. Liver lesions were induced in the mice by treatment with a single dose of diethylnitrosamine at 30 days of age 36 weeks before treatment with the phenobarbital. Three mice at each phenobarbital concentration were sampled at each time, and the labeling index was determined by autoradiography. A minimum of 25 adenomatous lesions were scored for the three mice. All cells in each adenoma were scored. (*) Values significantly greater than the untreated control ($p < 0.05$).

the lowest dose (20 mg/L) having no effect on the labeling index. This shows a correlation to the dose - response effect of phenobarbital with regard to hepatic cancer induction in mice. Treatment of mice with doses above 100 mg/L produced a significant increase in hepatic tumors in the mice. Studies of chronic treatment with phenobarbital at doses of 20 mg/L and lower have not been performed on mice. Therefore, a dose-response correlation between the selective induction of hepatic DNA synthesis by phenobarbital in the focal lesions and the induction of hepatic cancer has not been confirmed (*9,10*).

Based on these preliminary results, it appears that the hepatic foci uniquely respond to the DNA synthesis inductive effects of phenobarbital compared to the normal, surrounding hepatocytes and the adenomatous hepatocytes. Although the normal, surrounding hepatocytes showed a temporal dose-response increase in labeling index at the two highest doses studied, this labeling index returned to control levels after the 14-day treatment period. The hepatic foci showed a dose-responsive increase in labeling that remained elevated for the entire duration of the phenobarbital exposure. In contrast, the hepatic adenomas were refractory to the DNA synthesis inductive effects of phenobarbital at all the phenobarbital doses examined. It appears from these data that the adenoma stage of hepatic cancer may have been refractory to the effects of the nongenotoxic carcinogen (in this case phenobarbital).

The mechanism for the observed selective DNA synthesis response of the foci to phenobarbital remains to be resolved. It may be the result of intrinsic differences in the preneoplastic focal cell that allow for the increased response to phenobarbital. These intrinsic differences could be the result of differences in response to growth factors (*14,15*), hormones (such as the glucocorticoids), or metabolism of the phenobarbital. It should be emphasized that in the present study the cell proliferative rate and cell death rate in the hepatic lesions were not quantitated. Previous studies in the rat have shown that modulation of the cell death rate in hepatic lesions plays an important role in the selective cell proliferative-enhancing effect of phenobarbital on the hepatic foci (*16*). However, contrary to the results in the present study in the mouse, Schulte-Hermann et al. (*17*) showed that in the rat liver, phenobarbital increased cell growth but not through an increase in DNA synthesis. These workers noted that the increase was through a selective decrease in cell death rate in the hepatic foci. Whether this species difference in the effect of phenobarbital on focal lesion DNA synthesis is species dependent or protocol dependent remains unanswered. Attempts to quantitate the rate of apoptosis in the mouse liver lesions has proven difficult. Therefore, the effects of phenobarbital on focal lesion cell death rate and normal, surrounding hepatocyte death rate (measured by apoptosis) remains to be quantitated (*17*).

Studies on the mechanisms of why the preneoplastic foci display a selective response to phenobarbital remain to be performed. The system used in the present study provides us with a valuable model for examining the effects of extrinsic and intrinsic agents on the DNA synthesis and growth of hepatic tumors. This model may be of value in examining both growth stimulatory and growth inhibitory effects of various agents on hepatic preneoplastic and neoplastic lesions.

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